Computer modelling reveals new conformers of the ATP binding loop of Na$^+$/K$^+$-ATPase involved in the transphosphorylation process of the sodium pump

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ABSTRACT

Hydrolysis of ATP by Na$^+$/K$^+$-ATPase, a P-Type ATPase, catalyzing active Na$^+$ and K$^+$ transport through cellular membranes leads transiently to a phosphorylation of its catalytical α-subunit. Surprisingly, three-dimensional molecular structure analysis of P-type ATPases reveals that binding of ATP to the N-domain connected by a hinge to the P-domain is much too far away from the Asp^{369} to allow the transfer of ATP's terminal phosphate to its aspartyl-phosphorylation site. In order to get information for how the transfer of the γ-phosphate group of ATP to the Asp^{369} is achieved, analogous molecular modeling of the M$_4$–M$_5$ loop of ATPase was performed using the crystal data of Na$^+$/K$^+$-ATPase of different species. Analogous molecular modeling of the cytoplasmic loop between Thr^{338} and Ile^{760} of the α_2-subunit of Na$^+$/K$^+$-ATPase and the analysis of distances between the ATP binding site and phosphorylation site revealed the existence of two ATP binding sites in the open conformation; the first one close to Phe^{475} in the N-domain, the other one close to Asp^{369} in the P-domain. However, binding of Mg^{2+}•ATP to any of these sites in the "open conformation" may not lead to phosphorylation of Asp^{369}. Additional conformations of the cytoplasmic loop were found wobbling between "open conformation" <==> "semi-open conformation <==> "closed conformation" in the absence of 2Mg^{2+}•ATP. The cytoplasmic loop's conformational change to the "semi-open conformation"—characterized by a hydrogen bond between Arg^{543} and Asp^{611}—triggers by binding of 2Mg^{2+}•ATP to a single ATP site and conversion to the "closed conformation" the phosphorylation of Asp^{369} in the P-domain, and hence the start of Na$^+$/K$^+$-activated ATP hydrolysis.

Subjects Biochemistry, Bioinformatics, Biophysics, Computational Biology, Computational Science
Keywords M4M5 loop, Open and closed conformations, Hinge movement, Na$^+$/K$^+$-ATPase phosphorylation

INTRODUCTION

Na^+/K^+-ATPase (EC 3.6.3.9) is an integral membrane protein that transports sodium and potassium ions against an electrochemical gradient. It belongs to the family of P-type ATPases that is structurally typified by the L-2-haloacid dehalogenase. Na^+/K^+-ATPase and Ca^{2+}-ATPase belong to this family and show a high degree of homology, especially at the phosphorylation domain. The tertiary structure of Na^+/K^+-ATPase has been solved at high resolution by X-ray crystallography (*Kanai et al., 2013*; *Laursen et al., 2015*; *Laursen et al., 2013*; *Morth et al., 2011*; *Morth et al., 2007*; *Nyblom et al., 2013*; *Ogawa et al., 2015*; *Ogawa et al., 2009*; *Shinoda et al., 2009*; *Yatime et al., 2011*) and also partially several N-domain structures by X-ray crystallography (*Håkansson, 2003*) and NMR (*Mark Hilge et al., 2003*). In addition, several crystallographic structures of Ca^{2+}-ATPase were reported (*Akin et al., 2013*; *Bublitz et al., 2015*; *Clausen et al., 2013*; *Drachmann et al., 2014*; *Jensen et al., 2006*; *Laursen et al., 2009*; *MacLennan & Green, 2000*; *Moncoq, Trieber & Young, 2007*; *Obara et al., 2005*; *Olesen et al., 2007*; *Olesen et al., 2004*; *Paulsen et al., 2013*; *Sacchetto et al., 2012*; *Sohoel et al., 2006*; *Sonntag et al., 2011*; *Sorensen, Moller & Nissen, 2004*; *Takahashi, Kondou & Toyoshima, 2007*; *Toyoshima, 2008*; *Toyoshima et al., 2013*; *Toyoshima & Mizutani, 2004*; *Toyoshima et al., 2000*; *Toyoshima & Nomura, 2002*; *Winther et al., 2010*; *Winther et al., 2013*).

The Na^+/K^+-ATPase consists of three subunits, the catalytic α-subunit with a molecular mass of about 110 kDa, the β-subunit, a glycoprotein with the molecular mass of 40–60 kDa (neglecting the oligosaccharides) and eventually the associated γ-subunit with the molecular mass of 8–14 kDa (*Collins & Leszyk, 1987*; *Forbush, Kaplan & Hoffman, 1978*). The α-subunit carries out all ion transport and catalytic functions. The ion transport of Na^+ and K^+ catalyzed by Na^+/K^+-ATPase in this subunit is believed to occur via transition between two major conformational states, the E_1Na^+ and the E_2K^+-conformations (*Kaplan, 2002*). The α-subunit contains in a large cytoplasmic loop between the M_4 and M_5 transmembrane helices the catalytic center binding and hydrolyzing ATP. This large loop protruding far to cytoplasm comprises quite rigid subdomains and self-supporting substructures (*Amler, Abbott & Ball, 1992*). Structurally it consists of two main parts, the rigid nucleotide binding domain (N-domain) (*Kanai et al., 2013*; *Nyblom et al., 2013*) roughly between the amino acid residues Arg^{380}–Arg^{583}, and the domain forming the Asp^{369}-phosphointermediate during ATP hydrolysis (P-domain).

The secondary structure of N-domain shows a seven-stranded antiparallel β-sheet with two helix bundles sandwiching it. In this domain Phe^{548}, Glu^{505}, Lys^{501}, Gln^{482}, Lys^{480}, Ser^{477}, Phe^{475} and Glu^{446} participate in docking of the Mg^{2+}ATP complex into its binding pocket (*Kubala et al., 2003*).

The P-domain consists of two parts (subdomains). Its N-terminal subdomain ranges from Lys^{347} to the residue of phosphorylation Asp^{369}. It is connected to the fourth transmembrane segment M_4 of the α-subunit. A highly negatively charged surface was found around the phosphorylation site accessible by the solvent (*Tejral et al., 2007*; *Tejral et al., 2009*). The C-terminal subdomain formed by Ala^{590}–Phe^{747} is connected to the fifth transmembrane segment M_5. These two parts (subdomains) form a typical Rossmann

fold. The secondary structure of this domain can be divided into a seven-stranded parallel β-sheet with eight short associated helices (*Morth et al., 2007*; *Ogawa et al., 2009*; *Shinoda et al., 2009*).

Despite the relatively large amount of information available on the 3-D structure of Na^+/K^+-ATPase, the molecular mechanism of the transphosphorylation process of the terminal γ-phosphate group of ATP residing in the N-domain to the Asp^{369}-acceptor group at the P-domain is still a puzzle. Evidently, the N-domain must bend to the P-domain by way of a mobile hinge structure. It is not clear, however, how this process is achieved on a molecular level. Hence, we tried to get information on this question using molecular modeling.

METHODS

Comparative modeling of the open conformation

As the solved crystal structures of Na^+/K^+-ATPase are only of a non-human origin (*Håkansson, 2003*; *Kanai et al., 2013*; *Laursen et al., 2015*; *Laursen et al., 2013*; *Mark Hilge et al., 2003*; *Morth et al., 2011*; *Morth et al., 2007*; *Nyblom et al., 2013*; *Ogawa et al., 2015*; *Ogawa et al., 2009*; *Shinoda et al., 2009*; *Yatime et al., 2011*), we decided to employ the procedure of homology modeling to get its human α_2-subunit 3-D-structure. The primary amino acid sequence of the human Na^+/K^+-ATPase was retrieved from the ExPASy server (UniProt KB/TrEMBL; http://www.expasy.ch/). The resulting P50993 (AT1A2_HUMAN) target human sequence in the length of 1,020 amino acids for the Na^+/K^+-ATPase α_2-subunit precursor of sodium/potassium-transporting ATPase α_2-subunit, Homo sapiens, EC 3.6.3.9, was chosen (*Shull, Pugh & Lingrel, 1989*). Five amino acids at the N-terminal beginning of this sequence compared to the translated RNA sequence do not occur in the native form (*Hara et al., 1987*; *Kawakami et al., 1985*; *Ovchinnikov et al., 1986*; *Shull, Schwartz & Lingrel, 1985*). Hence, they were not included in our further numbering. For modeling, known structures of Na^+/K^+-ATPase deposited at the RCSB Protein Data Bank (http://www.pdb.org/) were used. In order to create the model based on the above mentioned sequence, the solved crystal structures of Na^+/K^+-ATPase with RCSB Protein Data Bank (http://www.pdb.org/) accession codes 3B8E (*Morth et al., 2007*) and 3KDP (*Morth et al., 2007*) were used as the templates for our modeling. The multialignment of the chosen target (P50993, AT1A2_HUMAN) sequence and the two templates (3B8E, 3KDP) for open conformation was prepared by MODELLER program (salign module) (*Eswar et al., 2006*; *Marti-Renom et al., 2000*; *Šali, 1995*; *Šali & Blundell, 1993*). The choice of templates (solved crystal structures) was based on the species proximity (pig over shark) and absence of any cardioglycosides in the solved crystal structure, in order to get as close to the native form as possible. Using this multialignment and the solved 3D crystal structures, we have generated thousand M_4M_5-loop models by the MODELLER (automodel module) (*Eswar et al., 2006*; *Marti-Renom et al., 2000*; *Šali, 1995*; *Šali & Blundell, 1993*) program. From those created models the best thirty were selected using the PROCHECK (*Laskowski et al., 1993*; *Morris et al., 1992*) and Verifi3D (*Bowie, Luthy & Eisenberg, 1991*; *Lüthy, Bowie & Eisenberg, 1992*) programs (Table 1).

Table 1 The assessment of homology model quality (compared to the ones of template crystal structures).

Model	Compound (pdb code)	Ramachandran plot: percent of aminoacids in allowed regions	Procheck: overall G-factor	Verify3D: percent of residues that had an averaged 3D-1D score $\geq$ 0.2	Total energy (kJ/mol) (GROMOS96)
Open conformation	3b8eA (crystal structure)	93.6	0.01	88.44	−4645.14
	3b8eC (crystal structure)	93.3	0.01	91.98	−4644.72
	3kdpA (crystal structure)	95.8	−0.31	92.69	−3484.95
	3kdpC (crystal structure)	96.3	−0.32	92.69	−2921.80
	Model of open conformation	98.2	−0.21	97.64	−14113.98
Closed conformation	3wguA (crystal structure)	98.9	−0.07	99.53	−10732.51
	3wguC (crystal structure)	99.8	0.01	97.17	−13461.95
	3wgvA (crystal structure)	98.7	−0.10	96.70	−10132.11
	3wgvC (crystal structure)	100.0	−0.02	95.99	−12870.88
	4hqjA (crystal structure)	99.2	0.10	98.58	−12983.13
	4hqjC (crystal structure)	99.4	0.13	98.58	−13081.49
	Model of closed conformation	99.5	0.06	96.93	−7995.71

Comparative modeling of the closed conformation

As in the previous comparative modeling procedure, we have used the sequence P50993 (AT1A2_HUMAN) for modeling of Na$^+$/K$^+$-ATPase in the closed conformation. However, the solved crystal structures of the RCSB Protein Data Bank (http://www.pdb.org/—accession codes 3WGU, 3WGV and 4HQJ (*Kanai et al., 2013*; *Nyblom et al., 2013*)) were used as the templates for our modeling. Using the above-mentioned settings for the modeling program (MODELLER see previous paragraph) we obtained ten models of Na$^+$/K$^+$-ATPase in the closed conformation. From these, the best model has been chosen using the above-mentioned PROCHEK and Verifi3D programs (Table 1).

Docking, using the open and closed conformations

The best thirty models corresponding to the open conformation and the best model for the closed conformation were used for docking of Mg^{2+}•ATP complex, using the Vina-Autodock program (*Trott & Olson, 2010*). We have decided to use the whole Mg^{2+}•ATP complex, which has been derived from structures containing ATP, deposited in RCSB Protein Data Bank (http://www.pdb.org/). The sequential docking of Mg^{2+}, followed by ATP, has not been used, since the bond between Mg^{2+} and ATP phosphates is stronger than between Mg^{2+} and –COOH groups of amino acids (*Alberty, 1969*; *Dudev, Cowan & Lim, 1999*). In addition, the same procedure was used for the model of the closed conformation.

Molecular dynamics

Molecular dynamics (MD) of the M$_4$M$_5$-loop were simulated by Gromacs (*Berendsen, Van der Spoel & Van Drunen, 1995*; *Hess et al., 2008*; *Lindahl, Hess & Van der Spoel, 2001*; *Van der Spoel et al., 2005*; *Van der Spoel et al., 2010*), using the OPLS-AA potential (*Jorgensen, Maxwell & Tirado-Rives, 1996*; *Jorgensen & Tirado-Rives, 1988*; *Kaminski et al., 2001*; *Meagher, Redman & Carlson, 2003*; *Pranata, Wierschke & Jorgensen, 1991*) with combination of water model TIP3P (*Jorgensen et al., 1983*). Our protein model with or without

$2Mg^{2+} \bullet ATP$ were put into a rectangular box with a 1 nm thick layer of the water molecules around and periodic boundary conditions (*Berendsen, Van der Spoel & Van Drunen, 1995*; *Hess et al., 2008*; *Lindahl, Hess & Van der Spoel, 2001*; *Van der Spoel et al., 2005*; *Van der Spoel et al., 2010*).

The PME method (*Darden, York & Pedersen, 1993*) with a length parameter of 1 nm was used to describe Coulomb type electrostatic interactions and the cut-off method with a length parameter of 1 nm for the calculation of van der Waals interactions. As the first step of the MD simulation, the system of protein and water was energetically optimized using the method of steepest descents, followed by a conjugate gradient minimization algorithm with maximum 2.5×10^4 steps and maximum force smaller than 10 kj mol^{-1} nm^{-1} as the convergence criterion (see Supplemental Information 1). The *Berendsen et al. (1984)* coupling method was employed for the temperature and pressure coupling of a system to reflect the reference temperature of 310 K and the pressure of 1 bar. The leap-frog integration with 10^4 steps was used for stabilization, with integration step of 1 fs, corresponding to 10 ps simulation time to reach the equilibrium of the rectangular box (see Supplemental Information 1). This stabilized rectangular box was used for the main thirty simulations with 5×10^6 steps (2 fs single step), corresponding to 10 ns for each stabilization using the same simulation parameters as for the box stabilization. These thirty trajectories were simulated with independently generated initial conditions corresponding to a Maxwell distribution for a temperature of 310 K. The translation and rotation around the center of mass of the protein were removed, avoiding thus the simulated system distortion in the simulation box. The molecular dynamics simulation of the Na$^+$/K$^+$-ATPase in semi-open conformation with docked $2Mg^{2+} \bullet ATP$ was carried out using the same parameters as described above.

Docking to the semi-open conformation

From the charts of the time evolution of distances, the typical trajectory for the molecular dynamics of the open conformation without $2Mg^{2+} \bullet ATP$ has been chosen. The model showing a distance between Asp^{369} and Phe^{475} smaller than 2.2 nm and with the best PROCHECK and Verify3D scores were taken for docking of the $2Mg^{2+} \bullet ATP$ complex.

RESULTS

Assembly of the static 3D computational model of Na$^+$/K$^+$-ATPase

The main goal of this work was to describe the molecular mechanism of γ-phosphate transfer from the ATP in the binding site to the phosphorylation site (Asp^{369}) of Na$^+$/K$^+$-ATPase. To achieve this, first, a static three-dimensional model of Na$^+$/K$^+$-ATPase was developed based on the latest data and information. The P50993 (AT1A2_HUMAN) human target sequence of the Na$^+$/K$^+$-ATPase α_2-isoform (sodium/potassium-transporting ATPase α_2-subunit, Homo sapiens) was used for modeling and 3D model assembling. Two templates of Na$^+$/K$^+$-ATPase structures of accession codes 3B8E (*Morth et al., 2007*) and 3KDP (*Morth et al., 2007*) were retrieved from the Protein Data Bank which were proposed by UniProt server as sequence P05024 (sodium/potassium-transporting ATPase α_1-subunit, Sus scrofa) with sequences identity 86.5% of the target sequence. The alignments for open
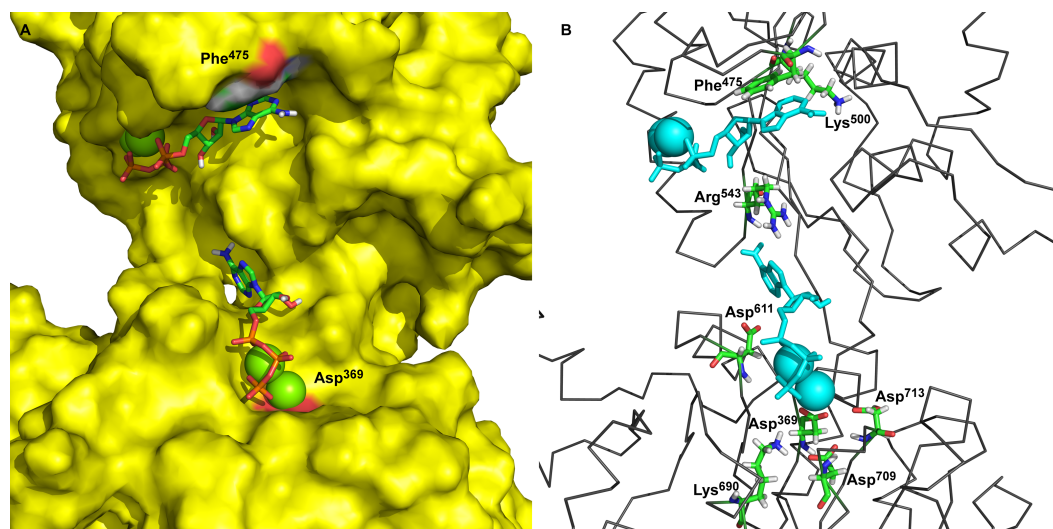
Figure 1 **Two ATP binding sites in "open" conformation.** (A) ATP bound near Phe[475] has docking energy -7.6 kcal/mol, ATP near Asp[369] has docking energy -8.9 kcal/mol. (B) The interacting amino acids with docked $2Mg^{2+}$ATP—both binding sites.

conformation of the M_4M_5-loop were prepared with identity of the 85.6% between the corresponding sequences of the human α_2-isoform and pig α_1-isoform for the M4M5-loop. The alignment and template structures were used for comparative modeling using the MODELLER program. The obtained 3D models were verified, applying the PROCHECK and Verifi3D programs (Table 1). Our modeling procedure resulted in static structures of the M_4M_5-loop of human α_2 isoform of Na^+/K^+-ATPase between Thr^{338} and Ile^{760} (see Fig. 1, with docked $2Mg^{2+}$•ATP). These models show distances around 3.26 nm between Phe[475] as part of the ATP-binding site and the α-carbon of Asp[369], the acceptor site for the phosphointermediate in ATP hydrolysis.

Two ATP binding sites exist in the open conformation of Na^+/K^+-ATPase

The obtained models of the open conformation of Na^+/K^+-ATPase were tested by an *in silico* ATP-docking experiment for its ability to bind Mg^{2+}•ATP. Surprisingly, we identified two possible docking sites (Fig. 1): the first one is in closest vicinity to Phe[475] ("the Phe[475] location") and the second one is close to Asp[369], ("the Asp[369] location"). Both binding sites showed only slightly different docking energies. While the docking energy at the Phe[475] location was $E_b = -7.6$ kcal/mol, the docking energy at the Asp[369] location was $E_b = -8.6$ kcal/mol. A closer insight into our model clearly indicated interactions among π-electrons between Phe[475] and the ATP adenine ring at the Phe[475] location, but the interaction between ATP's phosphates with bound magnesium and the negatively charged aspartate residue was responsible for the $2Mg^{2+}$•ATP binding at the Asp[369] location.

The amino acids found in the neighborhood of docked Mg^{2+}•ATP in both binding sites were identified and found to be in agreement with already published data (*Jacobsen, Pedersen & Jorgensen, 2002*; *Jorgensen, Hakansson & Karlish, 2003*; *Jorgensen, Jorgensen*
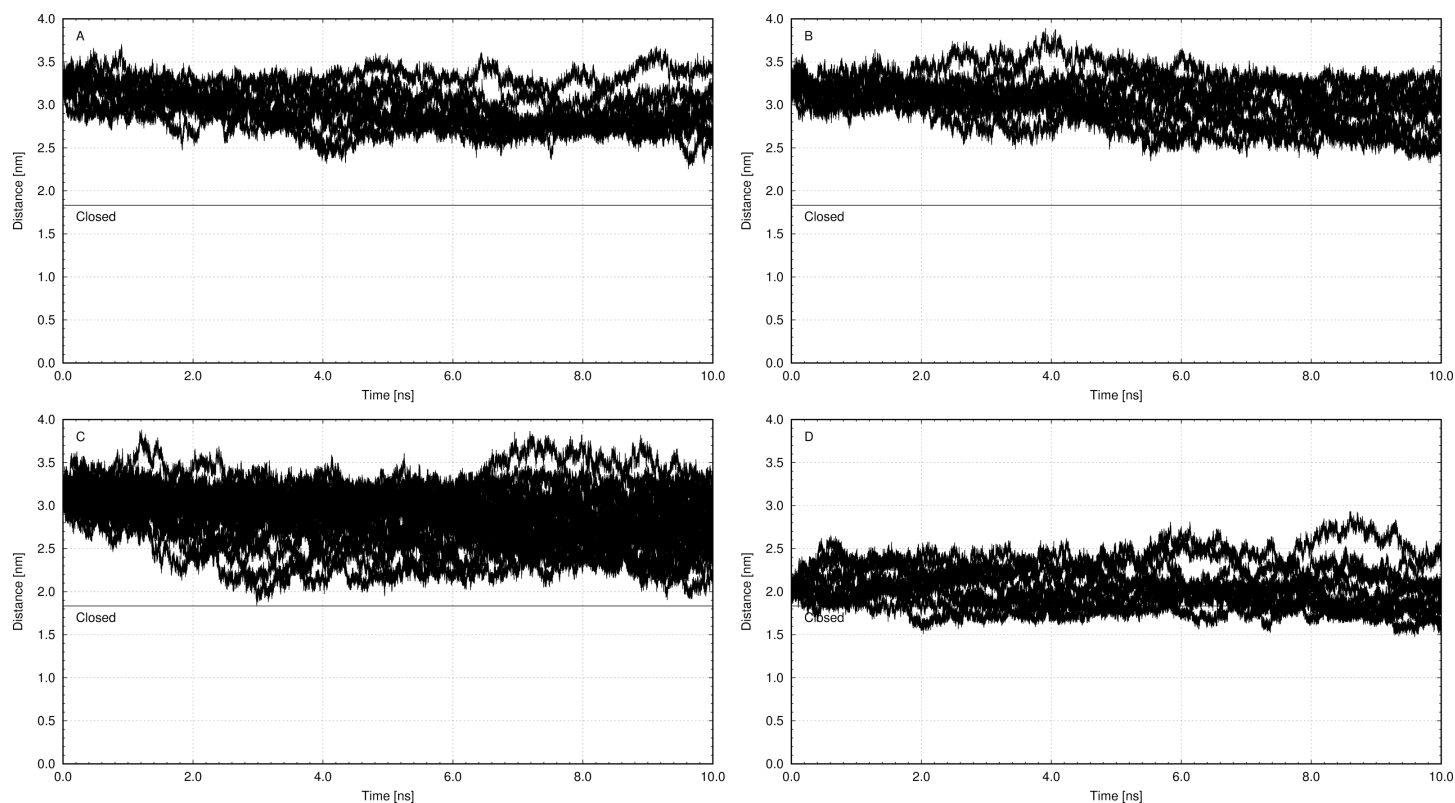
Figure 2 (A) Molecular dynamics simulation of the model in open state (change of distance between Phe[475] and Asp[369] during simulation) with $2Mg^{2+}$ATP interacting with Phe[475]. (B) Molecular dynamics simulation of the model in open state (change of distance between Phe[475] and Asp[369] during simulation) $2Mg^{2+}$ATP interacting with Asp[369]. (C) Molecular dynamics simulation of the model in open state (change of distance between Phe[475] and Asp[369] during simulation) without $2Mg^{2+}$ATP. (D) Molecular dynamics simulation of the model in open state (change of distance between Phe[475] and Asp[369] during simulation) with $2Mg^{2+}$ATP docked in semi-open conformation (C), and interacting with both Phe[475] and Asp[369] (closed conformation).

& Pedersen, 2001; Jorgensen & Pedersen, 2001; Kubala et al., 2003; Pedersen, Jorgensen & Jorgensen, 2000).

High ATP concentration hinders the enzyme cycle and keeps the Na$^+$/K$^+$-ATPase at the open conformation

The structure with best docking energy for both $2Mg^{2+}\bullet$ATP docking sites was the starting point for a molecular dynamics simulation, which revealed another surprising result. The molecular dynamics simulation in the presence of $2Mg^{2+}\bullet$ATP (we ran two simulations series, one with $2Mg^{2+}\bullet$ATP docked in the ATP binding site and the other in the phosphorylation site) did not result in a stable close conformation needed to phorphorylate Asp[369] during Na$^+$/K$^+$-ATP hydrolysis. Interestingly, the enzyme preferentially remained in the open conformation in both simulations as is evident from the resulting distance distribution between α-carbons of Asp[369] and Phe[475] (Figs. 2A, 2B, 3A and 3B, the distance varied from 2.5 nm to 3.4 nm, with maxima 2.9 nm and 3.1 nm respectively).

However, molecular dynamics experiments in the absence of $2Mg^{2+}\bullet$ATP (Fig. 3C) in nanosecond timescale exhibited a different pattern. This conformation was characterized
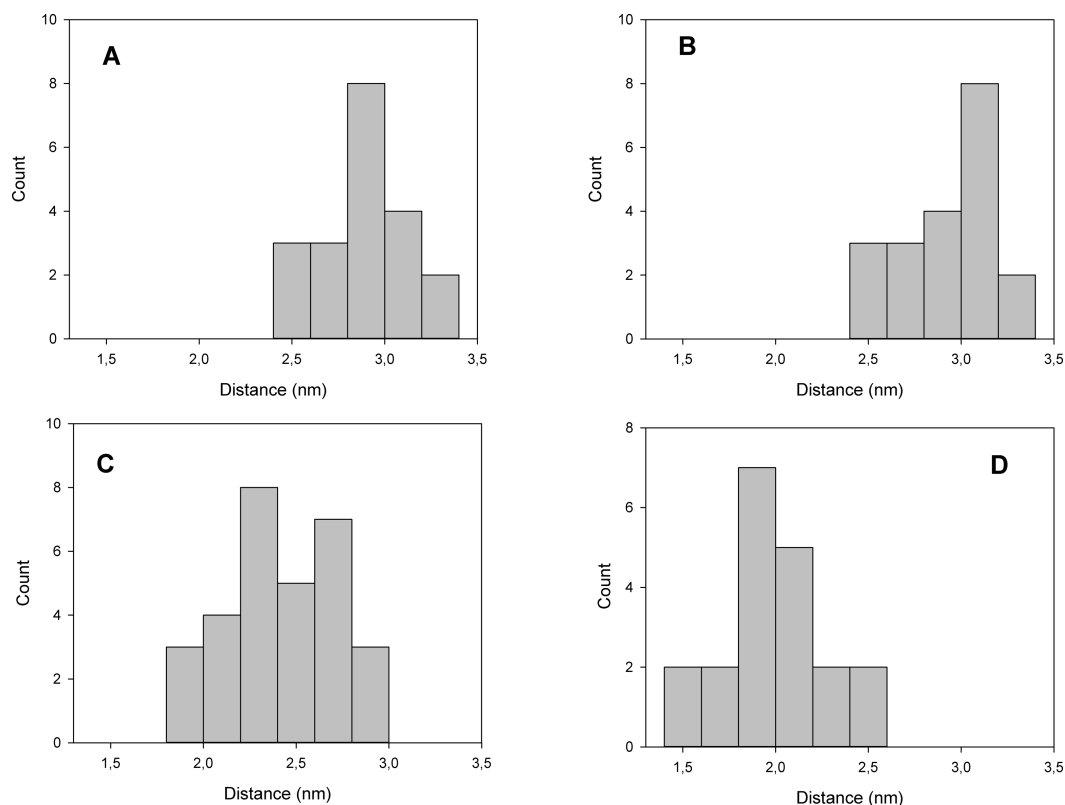
Figure 3 (A) Resulting distance distribution between Phe[475] and Asp[369] at the end of simulation with $2Mg^{2+}$ATP interacting with Phe[475]. (B) Resulting distance distribution between Phe[475] and Asp[369] at the end of simulation with $2Mg^{2+}$ATP interacting with Asp[369]. (C) Resulting distance distribution between Phe[475] and Asp[369] at the end of simulation without $2Mg^{2+}$ATP. (D) Resulting distance distribution between Phe[475] and Asp[369] at the end of simulation with $2Mg^{2+}$ATP docked in semi-open conformation (C), and interacting with both Phe[475] and Asp[369] (closed conformation).

by shortening of the distance between α-carbons of Asp[369] and Phe[475] to about $d \sim 2.00$ nm (Fig. 3C).

Additionally, we performed 30 simulations in the absence of $2Mg^{2+} \bullet$ATP. Yet, there was no stable result: sometimes, the molecular dynamic simulation led to the new conformation (we will call this conformation "semi-open" conformation), but sometimes the enzyme remained in the open conformation, with the ratio open/semi-open conformation being approximately 1:1.4 (Figs. 2C and 3C). Clearly, conformational transitions between the "open" and "semi-open conformations" seem to be rather a stochastic process (Figs. 2C and 3C). Consequently, we decided to call this newly identified conformation representing a distance of 2.3 nm between Phe[475] and Asp[369] the "semi-open" conformation of the catalytic site of Na^+/K^+-ATPase. The semi-open conformation is characterized by the formation of a hydrogen bond between Arg[543] and Asp[611] (see Fig. 4).
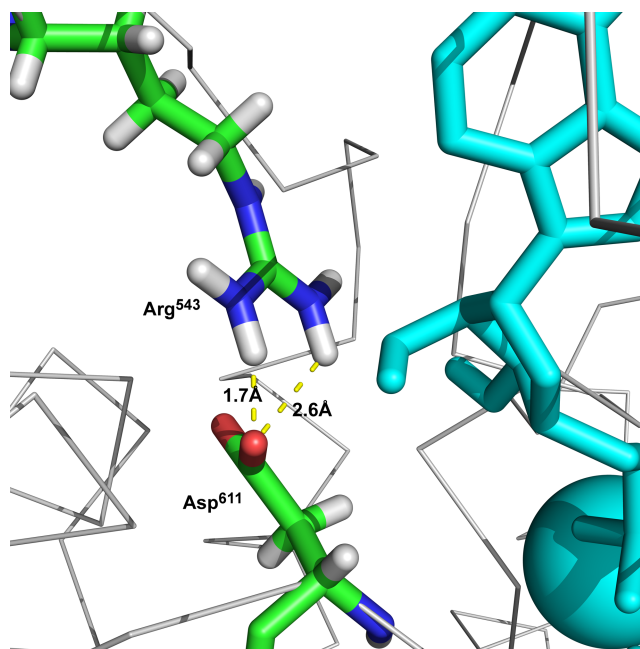
Figure 4 **The hydrogen bonds between Arg[543] and Asp[611] formed in semi-open state.**

## Hinge movement, ATP binding and enzyme phosphorylation

Release and re-binding of $2Mg^{2+} \bullet$ATP complexes at two different binding sites in the open conformation may have huge consequences for the molecular mechanism of the transphosphorylation process to Asp[369] as part of the ATP hydrolysis of Na$^+$/K$^+$-ATPase. Most importantly is the fact that the α-subunit of Na$^+$/K-ATPase can wobble between the "open" and "semi-open conformations" in the absence of $2Mg^{2+} \bullet$ATP.

Naturally, the obvious question arises, whether and how the $2Mg^{2+} \bullet$ATP complex interacts with the "semi-open conformation." Therefore, $2Mg^{2+} \bullet$ATP molecule has been docked into the "semi-open conformation" (Fig. 5), revealing only a single $2Mg^{2+} \bullet$ATP binding site exists. This $2Mg^{2+} \bullet$ATP binding site in the "semi-open conformation" was formed as a sandwich structure from both, "the Phe[475] location" and "the Asp[369] location" as they were revealed and identified at the open conformation. Both sites have approached each other due the stochastic process in the absence of $2Mg^{2+} \bullet$ATP, probably due to the preceding hinge movement in the absence of $2Mg^{2+} \bullet$ATP. This binding pocket for a single $2Mg^{2+} \bullet$ATP is characterized by the most favorable and highest docking energy of $E_b = -8.8$ kcal/mol.

Furthermore, docking of $2Mg^{2+} \bullet$ATP to the "semi-open conformation" results in a further substantial shortening of the distance between the Asp[369] and Phe[475]. Consequently, the γ-phosphate of $2Mg^{2+} \bullet$ATP was attracted to Asp[369] and the mutual distance between the α-carbons of Asp[369] and Phe[475] decreased to approximately $d = 1.8$ nm (Figs. 2D and 3D, majority falling in the interval 1.5–2.0 nm). This shortening can be explained as a consequence of the second phase of the hinge movement: bending of the N-domain toward the P-domain, which completes "the hinge mechanism" (Fig. 6).
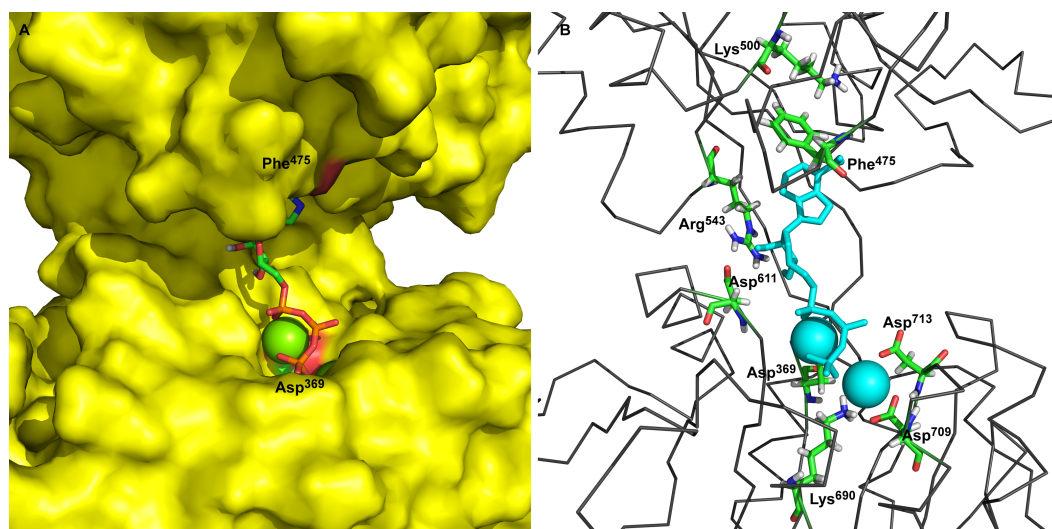
Figure 5 Docking of 2Mg²⁺•ATP to the "semi-open" conformation. The simultaneous interaction of $2Mg^{2+}•ATP$ with Phe^{475} and Asp^{369} can be identified.
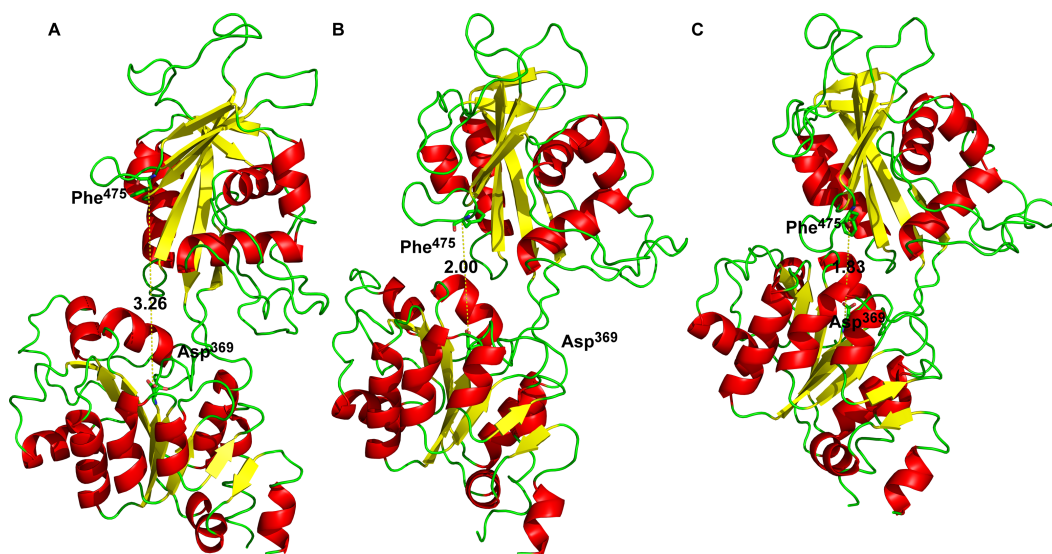
Figure 6 The three conformational states of Na⁺/K⁺-ATPase (distances are in Å). From the left: (A) Open conformation, (B) "Semi-open" conformation and (C) Closed conformation.

In order to verify our conclusions, the closed conformation of Na⁺/K⁺-ATPase structure was prepared by homology modeling as well using crystallography templates (Fig. 7). The docking experiment of $2Mg^{2+}•ATP$ to the "closed" conformation, revealed the existence of a single ATP binding site as well (Fig. 7). Moreover, the molecular dynamic experiment with the "semi-open" sub-conformation shows that it's conformation differs from that one of the "closed" conformation with overall RMSD < 0.3 nm, which is within the experimental error of crystallographic data.
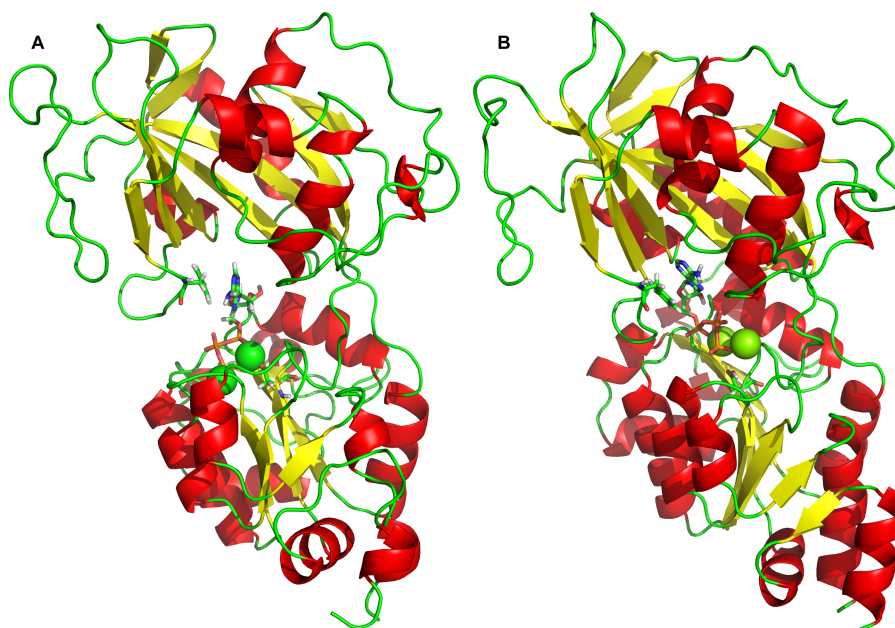
Figure 7 (A) The conformation received as the result of the molecular dynamic experiment with docked $2Mg^{2+} \bullet ATP$ (closed state). (B) The conformation received as the result of the homology modeling of Na^+/K^+-ATPase in the "closed" state, with docked $2Mg^{2+}ATP$. The overall difference between these two conformations has RMSD $= 0.27$ nm, which is within the error of the crystallographic data.

DISCUSSION

Multiple ATP binding sites are found in the open and semi-open conformations of the cytoplasmic M_4–M_5-loop of Na^+/K^+-ATPase

The intention of this work was to learn by inspection of a large number of related and crystallized P-type ATPases and analogous computer modeling of the cytoplasmic M_4–M_5-loop of the human α_2 isoform of Na^+/K^+-ATPase, how on a molecular level the distance is shortened between the nucleotide binding site (the N-domain) and the phosphorylation site Asp^{369} at the P-domain. The distance of 3.26 nm between both sites (in the "open" state, Fig. 6) is too high to support either the $Na^+ + Mg^{2+}$ or the Mg^{2+}-dependent transphosphorylation process or the ATP–ADP exchange reaction (*Fahn, Koval & Albers, 1966*), both the partial reactions of Na^+/K^+-ATPase. Evidently, any changes by binding of Na^+ or K^+ to their respective membrane sites must be excluded, since our analysis was restricted exclusively to the molecular events at the large cytoplasmic M_4–M_5-loop: We intended to understand the bending mechanism of the N-domain towards the P-domain.

ATP binding into the semi-open conformation leads to the hinge movement and triggers enzyme phosphorylation

We identified by molecular modeling of the cytoplasmic loop structure the existence of 3 different conformational states with the ability to bind ATP (Fig. 6). In the absence of ATP and $2Mg^{2+} \bullet ATP$ the "open" and "semi-open" conformational states are freely interconverted. The open state binds ATP in the absence of Mg^{2+} to the N-domain as previously shown (References). It may bind, however, also $2Mg2^+ \bullet ATP$ at 2 sites, the "the Phe^{475}

location'' and the ''the Asp^{369} location'' (Fig. 1). Yet, in this open conformation, no transphosphorylation of the gamma phosphate group of ATP to Asp^{369} residing on the P-domain is possible: the terminal phosphate of ATP is much too remote from the carboxyl group of Asp^{369}. Molecular modeling clearly showed that it is rather the newly identified semi-open conformation which binds $2Mg^{2+}\bullet ATP$ to a single site in such a way that the terminal phosphate approaches the phosphate acceptor site Asp^{369} on the P-domain leads and via a further shift to the ''occluded'' state may achieve its phosphorylation.

On a molecular level our model describes and is in agreement with the vast majority of published structures for the ATP binding domains of P-type ATPases (*Bublitz et al., 2013*; *Bueno-Orovio et al., 2014*; *Castillo et al., 2011*; *Castillo et al., 2015*; *Fuller et al., 2013*; *Howland, 1991*; *Jacobsen, Pedersen & Jorgensen, 2002*; *Jensen et al., 2006*; *Jorgensen, Hakansson & Karlish, 2003*; *Jorgensen, Jorgensen & Pedersen, 2001*; *Jorgensen & Pedersen, 2001*; *Kanai et al., 2013*; *Kaplan, 2002*; *Laursen et al., 2015*; *MacLennan & Green, 2000*; *Morth et al., 2007*; *Nyblom et al., 2013*; *Obara et al., 2005*; *Ogawa et al., 2009*; *Olesen et al., 2007*; *Pedersen, Jorgensen & Jorgensen, 2000*; *Sacchetto et al., 2012*; *Shinoda et al., 2009*; *Toyoshima, 2008*; *Toyoshima et al., 2013*; *Toyoshima & Mizutani, 2004*; *Toyoshima et al., 2000*). Importantly, Arg^{543} is located in the N domain near the interface to the P domain (Fig. 1). This residue has been shown to be essential for nucleotide binding; its substitution by Gln abolishes high-affinity binding of ATP (in the absence of Mg^{2+}) and also Na^+/K^+-ATPase activity (*Pedersen, Jorgensen & Jorgensen, 2000*). The free energy required overcoming the electrostatic interactions between the γ-phosphate of $2Mg^{2+}\bullet ATP$ and the carboxylate groups amounts to 7.9 kcal/mol for Asp^{369}. This value supports our model exactly. In addition, the increased binding energy of $2Mg^{2+}\bullet ATP$ is connected with a conformational transition constituting the driving force for transport of K^+ across the membrane (*Howland, 1991*). Additionally, our molecular modeling experiments showed in docking experiments a very favorable binding energy of $2Mg^{2+}ATP$ at the semi-open conformation. The strong electrostatic interaction with the negative charges of Asp^{369}, Asp^{709} and Asp^{713} with $2Mg^{2+}\bullet ATP$ shows that the γ-phosphate of the tightly bound ATP are important to approach the surface of the P domain in Na^+/K^+-ATPase (*Jorgensen, Hakansson & Karlish, 2003*; *Jorgensen, Jorgensen & Pedersen, 2001*; *Jorgensen & Pedersen, 2001*). This certainly leads to further bridging the gap between the N- and P-domains and the formation of a ''closed conformation'' (Fig. 6C) resulting in a type of ''occluded $2Mg^{2+}\bullet ATP$'' preceding the formation of a phosphointermediate in the ATP-E_1 form of the α subunit of Na^+/K^+-ATPase.

Analogously, in the crystal structure of Ca^{2+}-ATPase in the $E_1[2Ca^{2+}-]$ form (*Clausen et al., 2013*; *Toyoshima et al., 2013*; *Winther et al., 2013*), the N domain is separated from the P domain by a distance of 2.0–2.5 nm. Such a distance is also seen in our model in the ''open'' conformational state: the P domain of the human α_2 isoform of Na^+/K^+-ATPase is separated from the N domain by a distance of less than 2 nm. Additionally, Lys^{690} appears to create a salt linkage with the phosphate group as has been found in previous experiments. Mg^{2+} is essential for all phosphoryl transfer reactions. The experience from Mg^{2+} binding studies is that the binding affinity and the coordination pattern depend strongly on the conformational state (*Pedersen, Jorgensen & Jorgensen, 2000*) . Our model shows this as well. Importantly, fluctuation in between the ''open'' and ''semi-open
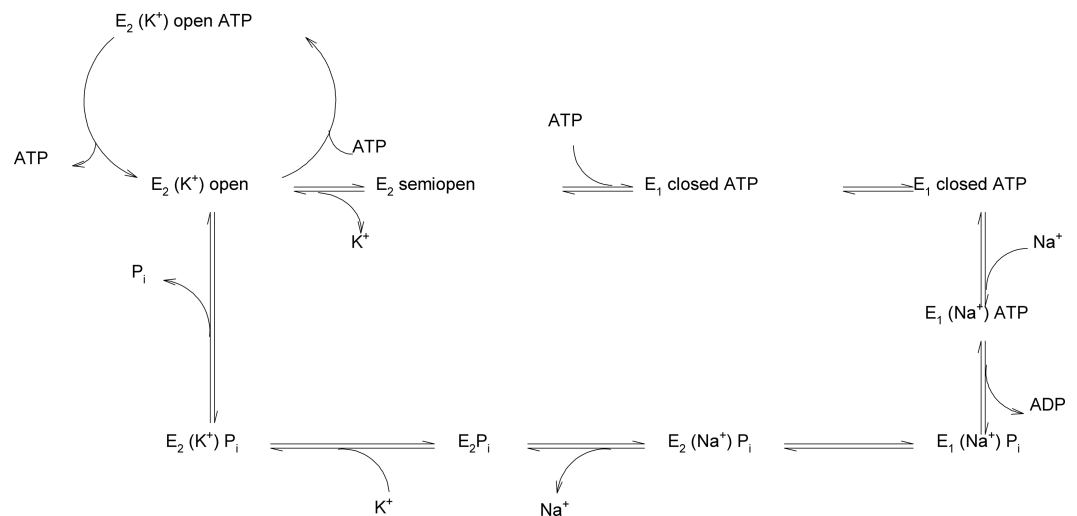
Figure 8 Reaction scheme of Na$^+$/K$^+$-ATPase explaining the possible functions of the "open", "semi-open" and "closed" conformations of the big cytoplasmic loop within the export of Na$^+$ and import of K$^+$ by the sodium pump.

conformations" is connected with binding/unbinding of 2Mg^{2+}•ATP to the three above mentioned negatively charged residues of Asp^{709}, Asp^{713} and Asp^{369} (Figs. 1B and 4). However, binding of 2Mg^{2+}•ATP to any site of the "open conformation" cannot lead to phosphorylation. The phosphorylation process can be triggered only when the "semi-open conformation" in the absence of ATP is formed. Once the semi-open conformation has been created, by forming a hydrogen bond between Arg^{543} and Asp^{611} (Fig. 4), the affinity for ATP peaks, facilitating thus ATP binding. The distance between Phe^{475} and Asp^{369} decreased to about 1.8 nm (corresponding to 1.83 in our "closed conformation" model). This average value of the experimentally reported distances for the "closed conformation", enables the phosphorylation of Asp^{369}, and is in accordance with the measured data (*Jacobsen, Pedersen & Jorgensen, 2002*; *Jorgensen, Jorgensen & Pedersen, 2001*).

   To the best of our knowledge, this is the first report on the existence of three conformers of the big cytoplasmic loop binding ATP. Our finding may have important consequences for understanding the molecular mechanism of the Na$^+$/K$^+$-ATPase function. Na$^+$ ions have been reported to increase the activity of transphosphorylation process (*Kaplan, 2002*). It is unclear at present, where Na$^+$ binds to its transport site in the transmembranal part of the enzyme and how this may affect the conformational transitions of the hinge region in the closing process approaching N- and P-domains such a way that the phosporylation of Asp^{369} as an intermediate may happen. It needs to be investigated in further studies, how, at a molecular level, high ATP concentrations lead to the release of E$_2$-occluded K$^+$. Micromolar ATP concentrations are sufficient for ATP binding (in the absence of Mg^{2+}) to the N-domain in the open state (*Kubala et al., 2003*; *Schoner, Beusch & Kramer, 1968*; *Tran & Farley, 1999*). The effect of Mg^{2+} on the binding of ATP to the isolated N-domain has never been studied. Micromolar ATP concentrations are sufficient for the Na$^+$ + Mg^{2+}-dependent formation of the Asp^{369}-phosphointermediate (*Hegyvary & Post, 1971*;

Moczydlowski & Fortes, 1981). Millimolar ATP concentrations are necessary for the overall Na^+/K^+-activated ATP hydrolysis necessary for Na^+/K^+-transport. High (millimolar) ATP concentrations are necessary to result in the de-occlusion of K^+ from its transmembrane site. Might it be that the existence of 2 ATP sites in the "open conformation" of the cytoplasmic loop (Fig. 1) represents a situation of opening of the closed catalytic site for MgATP at high concentrations of the energy substrate. It is well known that K^+ ions are on its way from the outside to the inside of the cell included into the transmembrane part of Na^+/K^+-ATPase. High concentrations of MgATP are necessary to release occludes K^+ from the sodium pump into the cytoplasm. One may speculate that binding of MgATP at millimolar concentrations may lead to a shift of the "closed conformation" to the "open conformation," i.e., the displacement of the N-domain via the hinge mechanism from the P-domain due to binding of millimolar MgATP to the N-domain (Fig. 8).

ADDITIONAL INFORMATION AND DECLARATIONS

Competing Interests
The authors declare there are no competing interests.

Author Contributions
- Gracian Tejral conceived and designed the experiments, performed the experiments, wrote the paper, prepared figures and/or tables.

- Bruno Sopko conceived and designed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables.
- Alois Necas wrote the paper, reviewed drafts of the paper.
- Wilhelm Schoner and Evzen Amler conceived and designed the experiments, wrote the paper, reviewed drafts of the paper.

Data Availability

The following information was supplied regarding data availability:

Comparative modeling of the different conformations are detailed in 'Methods'.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.3087#supplemental-information.

REFERENCES

Akin BL, Hurley TD, Chen Z, Jones LR. 2013. The structural basis for phospholamban inhibition of the calcium pump in sarcoplasmic reticulum. *Journal of Biological Chemistry* **288**:30181–30191 DOI 10.1074/jbc.M113.501585.

Alberty RA. 1969. Standard Gibbs free energy, enthalpy, and entropy changes as a function of pH and pMg for several reactions involving adenosine phosphates. *Journal of Biological Chemistry* **244**:3290–3302.

Amler E, Abbott A, Ball WJ. 1992. Structural dynamics and oligomeric interactions of Na^+, K(+)-ATPase as monitored using fluorescence energy transfer. *Biophysical Journal* **61**:553–568 DOI 10.1016/S0006-3495(92)81859-3.

Berendsen HJC, Postma JPM, Van Gunsteren WF, DiNola A, Haak JR. 1984. Molecular dynamics with coupling to an external bath. *The Journal of Chemical Physics* **81**:3684–3690 DOI 10.1063/1.448118.

Berendsen HJC, Van der Spoel D, Van Drunen R. 1995. GROMACS: a message-passing parallel molecular dynamics implementation. *Computer Physics Communications* **91**:43–56 DOI 10.1016/0010-4655(95)00042-E.

Bowie JU, Luthy R, Eisenberg D. 1991. A method to identify protein sequences that fold into a known three-dimensional structure. *Science* **253**:164–170 DOI 10.1126/science.1853201.

Bublitz M, Musgaard M, Poulsen H, Thogersen L, Olesen C, Schiott B, Morth JP, Moller JV, Nissen P. 2013. Ion pathways in the sarcoplasmic reticulum Ca2+-ATPase. *Journal of Biological Chemistry* **288**:10759–10765 DOI 10.1074/jbc.R112.436550.

Bublitz M, Nass K, Drachmann ND, Markvardsen AJ, Gutmann MJ, Barends TRM, Mattle D, Shoeman RL, Doak RB, Boutet S, Messerschmidt M, Seibert MM, Williams GJ, Foucar L, Reinhard L, Sitsel O, Gregersen JL, Clausen JD, Boesen T, Gotfryd K, Wang K-T, Olesen C, Møller JV, Nissen P, Schlichting I. 2015. Structural studies of P-type ATPase–ligand complexes using an X-ray free-electron laser. *IUCrJ* **2**:409–420 DOI 10.1107/S2052252515008969.

Bueno-Orovio A, Sanchez C, Pueyo E, Rodriguez B. 2014. Na/K pump regulation of cardiac repolarization: insights from a systems biology approach. *Pflugers Archiv* **466**:183–193 DOI 10.1007/s00424-013-1293-1.

Castillo JP, De Giorgis D, Basilio D, Gadsby DC, Rosenthal JJ, Latorre R, Holmgren M, Bezanilla F. 2011. Energy landscape of the reactions governing the Na$^+$ deeply occluded state of the Na$^+$/K$^+$-ATPase in the giant axon of the Humboldt squid. *Proceedings of the National Academy of Sciences of the United States of America* **108**:20556–20561 DOI 10.1073/pnas.1116439108.

Castillo JP, Rui H, Basilio D, Das A, Roux B, Latorre R, Bezanilla F, Holmgren M. 2015. Mechanism of potassium ion uptake by the Na(+)/K(+)-ATPase. *Nature Communications* **6**:7622 DOI 10.1038/ncomms8622.

Clausen JD, Bublitz M, Arnou B, Montigny C, Jaxel C, Møller JV, Nissen P, Andersen JP, Le Maire M. 2013. SERCA mutant E309Q binds two Ca^{2+} ions but adopts a catalytically incompetent conformation: structure and function of SERCA mutant E309Q. *The EMBO Journal* **32**:3231–3243 DOI 10.1038/emboj.2013.250.

Collins JH, Leszyk J. 1987. ". gamma. subunit" of sodium-potassium-ATPase, a small, amphiphilic protein with a unique amino acid sequence. *Biochemistry* **26**:8665–8668 DOI 10.1021/bi00400a026.

Darden T, York D, Pedersen L. 1993. Particle mesh Ewald: an N.log(N) method for Ewald sums in large systems. *The Journal of Chemical Physics* **98**:10089–10092 DOI 10.1063/1.464397.

Drachmann ND, Olesen C, Møller JV, Guo Z, Nissen P, Bublitz M. 2014. Comparing crystal structures of Ca2+-ATPase in the presence of different lipids. *FEBS Journal* **281**:4249–4262 DOI 10.1111/febs.12957.

Dudev T, Cowan JA, Lim C. 1999. competitive binding in magnesium coordination chemistry: water versus ligands of biological interest. *Journal of the American Chemical Society* **121**:7665–7673 DOI 10.1021/ja984470t.

Eswar N, Webb B, Marti-Renom MA, Madhusudhan MS, Eramian D, Shen MY, Pieper U, Sali A. 2006. Comparative protein structure modeling using Modeller. *Current Protocols in Bioinformatics* **Chapter 5(Unit 5)**:5.6.1–5.6.30 DOI 10.1002/0471250953.bi0506s15.

Fahn S, Koval GJ, Albers RW. 1966. Sodium-potassium-activated adenosine triphosphatase of electrophorus electric organ I. An Associated sodium-activated transphosphorylation. *Journal of Biological Chemistry* **241**:1882–1889.

Forbush III B, Kaplan JH, Hoffman JF. 1978. Characterization of a new photoaffinity derivative of ouabain: labeling of the large polypeptide and of a proteolipid component of the (sodium-potassium ion)-dependent ATPase. *Biochemistry* **17**:3667–3676 DOI 10.1021/bi00610a037.

Fuller W, Tulloch LB, Shattock MJ, Calaghan SC, Howie J, Wypijewski KJ. 2013. Regulation of the cardiac sodium pump. *Cellular and Molecular Life Science* **70**:1357–1380 DOI 10.1007/s00018-012-1134-y.

Håkansson O. 2003. The crystallographic structure of Na, K-ATPase N-domain at 2.6 Å resolution. *Journal of Molecular Biology* **332**:1175–1182 DOI 10.1016/j.jmb.2003.07.012.

Hara Y, Urayama O, Kawakami K, Nojima H, Nagamune H, Kojima T, Ohta T, Nagano K, Nakao M. 1987. Primary structures of two types of alpha-subunit of rat brain Na$^+$, K$^+$-ATPase deduced from cDNA sequences. *The Journal of Biochemistry* **102**:43–58 DOI 10.1093/oxfordjournals.jbchem.a122039.

Hegyvary C, Post RL. 1971. Binding of adenosine triphosphate to sodium and potassium ion-stimulated adenosine triphosphatase. *Journal of Biological Chemistry* **246**:5234–5240.

Hess B, Kutzner C, Van der Spoel D, Lindahl E. 2008. GROMACS 4: algorithms for highly efficient, load-balanced, and scalable molecular simulation. *Journal of Chemical Theory and Computation* **4**:435–447 DOI 10.1021/ct700301q.

Howland JL. 1991. Electrogenic ion pumps by Peter Läuger [Abstract 243]. *Biochemical Education* **20** DOI 10.1016/0307-4412(92)90213-6.

Jacobsen MD, Pedersen PA, Jorgensen PL. 2002. Importance of Na, K-ATPase residue alpha 1-Arg544 in the segment Arg544–Asp567 for high-affinity binding of ATP, ADP, or MgATP. *Biochemistry* **41**:1451–1456 DOI 10.1021/bi015891h.

Jensen A-ML, Sørensen TL-M, Olesen C, Møller JV, Nissen P. 2006. Modulatory and catalytic modes of ATP binding by the calcium pump. *The EMBO Journal* **25**:2305–2314 DOI 10.1038/sj.emboj.7601135.

Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. 1983. Comparison of simple potential functions for simulating liquid water. *The Journal of Chemical Physics* **79**:926–935 DOI 10.1063/1.445869.

Jorgensen PL, Hakansson KO, Karlish SJ. 2003. Structure and mechanism of Na, K-ATPase: functional sites and their interactions. *Annual Review of Physiology* **65**:817–849 DOI 10.1146/annurev.physiol.65.092101.142558.

Jorgensen PL, Jorgensen JR, Pedersen PA. 2001. Role of conserved TGDGVND-loop in Mg2+ binding, phosphorylation, and energy transfer in Na, K-ATPase. *Journal of Bioenergetics and Biomembranes* **33**:367–377 DOI 10.1023/A:1010611322024.

Jorgensen WL, Maxwell DS, Tirado-Rives J. 1996. Development and testing of the opls all-atom force field on conformational energetics and properties of organic liquids. *Journal of the American Chemical Society* **118**:11225–11236 DOI 10.1021/ja9621760.

Jorgensen PL, Pedersen PA. 2001. Structure-function relationships of Na$^+$, K$^+$, ATP, or Mg2+ binding and energy transduction in Na, K-ATPase. *Biochimica et Biophysica Acta (BBA)—Bioenergetics* **1505**:57–74 DOI 10.1016/S0005-2728(00)00277-2.

Jorgensen WL, Tirado-Rives J. 1988. The OPLS (optimized potentials for liquid simulations) potential functions for proteins, energy minimizations for crystals of cyclic peptides and crambin. *Journal of the American Chemical Society* **110**:1657–1666 DOI 10.1021/ja00214a001.

Kaminski GA, Friesner RA, Tirado-Rives J, Jorgensen WL. 2001. Evaluation and reparametrization of the OPLS-AA force field for proteins via comparison with

accurate quantum chemical calculations on peptides†. *The Journal of Physical Chemistry B* **105**:6474–6487 DOI 10.1021/jp003919d.

Kanai R, Ogawa H, Vilsen B, Cornelius F, Toyoshima C. 2013. Crystal structure of a Na$^+$-bound Na$^+$, K$^+$-ATPase preceding the E1P state. *Nature* **502**:201–206 DOI 10.1038/nature12578.

Kaplan JH. 2002. Biochemistry of Na, K-ATPase. *Annual Review of Biochemistry* **71**:511–535 DOI 10.1146/annurev.biochem.71.102201.141218.

Kawakami K, Noguchi S, Noda M, Takahashi H, Ohta T, Kawamura M, Nojima H, Nagano K, Hirose T, Inayama S, Hayashida H, Miyata T, Numa S. 1985. Primary structure of the *alpha*-subunit of Torpedo californica (Na$^+$ + K$^+$) ATPase deduced from cDNA sequence. *Nature* **316**:733–736 DOI 10.1038/316733a0.

Kubala M, Teisinger J, Ettrich R, Hofbauerova K, Kopecky V, Baumruk Jr V, Krumscheid R, Plasek J, Schoner W, Amler E. 2003. Eight amino acids form the ATP recognition site of Na(+)/K(+)-ATPase. *Biochemistry* **42**:6446–6452 DOI 10.1021/bi034162u.

Laskowski RA, MacArthur MW, Moss DS, Thornton JM. 1993. PROCHECK: a program to check the stereochemical quality of protein structures. *Journal of Applied Crystallography* **26**:283–291 DOI 10.1107/S0021889892009944.

Laursen M, Bublitz M, Moncoq K, Olesen C, Møller JV, Young HS, Nissen P, Morth JP. 2009. Cyclopiazonic acid is complexed to a divalent metal ion when bound to the sarcoplasmic reticulum Ca2+-ATPase. *Journal of Biological Chemistry* **284**:13513–13518 DOI 10.1074/jbc.C900031200.

Laursen M, Gregersen JL, Yatime L, Nissen P, Fedosova NU. 2015. Structures and characterization of digoxin- and bufalin-bound Na$^+$, K$^+$-ATPase compared with the ouabain-bound complex. *Proceedings of the National Academy of Sciences of the United States of America* **112**:1755–1760 DOI 10.1073/pnas.1422997112.

Laursen M, Yatime L, Nissen P, Fedosova NU. 2013. Crystal structure of the high-affinity Na$^+$K$^+$-ATPase-ouabain complex with Mg2+ bound in the cation binding site. *Proceedings of the National Academy of Sciences of the United States of America* **110**:10958–10963 DOI 10.1073/pnas.1222308110.

Lindahl E, Hess B, Van der Spoel D. 2001. GROMACS 3.0: a package for molecular simulation and trajectory analysis. *Journal of Molecular Modeling* **7**:306–317 DOI 10.1007/s008940100045.

Lüthy R, Bowie JU, Eisenberg D. 1992. Assessment of protein models with three-dimensional profiles. *Nature* **356**:83–85.

MacLennan DH, Green NM. 2000. Structural biology: pumping ions. *Nature* **405**:633–634 DOI 10.1038/35015206.

Mark Hilge GS, Vuister GW, Güntert P, Gloor SM, Abrahams JP. 2003. ATP-induced conformational changes of the nucleotide-binding domain of Na, K-ATPase. *Nature Structural Biology* **10**:468–474 DOI 10.1038/nsb924.

Marti-Renom MA, Stuart AC, Fiser A, Sanchez R, Melo F, Sali A. 2000. Comparative protein structure modeling of genes and genomes. *Annual Review of Biophysics and Biomolecular Structure* **29**:291–325 DOI 10.1146/annurev.biophys.29.1.291.

Meagher KL, Redman LT, Carlson HA. 2003. Development of polyphosphate parameters for use with the AMBER force field. *Journal of Computational Chemistry* **24**:1016–1025 DOI 10.1002/jcc.10262.

Moczydlowski EG, Fortes PA. 1981. Inhibition of sodium and potassium adenosine triphosphatase by 2′, 3′-O-(2,4,6-trinitrocyclohexadienylidene) adenine nucleotides. Implications for the structure and mechanism of the Na:K pump. *Journal of Biological Chemistry* **256**:2357–2366.

Moncoq K, Trieber CA, Young HS. 2007. The molecular basis for cyclopiazonic acid inhibition of the sarcoplasmic reticulum calcium pump. *Journal of Biological Chemistry* **282**:9748–9757 DOI 10.1074/jbc.M611653200.

Morris AL, MacArthur MW, Hutchinson EG, Thornton JM. 1992. Stereochemical quality of protein structure coordinates. *Proteins: Structure, Function, and Bioinformatics* **12**:345–364 DOI 10.1002/prot.340120407.

Morth JP, Pedersen BP, Buch-Pedersen MJ, Andersen JP, Vilsen B, Palmgren MG, Nissen P. 2011. A structural overview of the plasma membrane Na^+, K^+-ATPase and H+-ATPase ion pumps. *Nature Reviews Molecular Cell Biology* **12**:60–70 DOI 10.1038/nrm3031.

Morth JP, Pedersen BP, Toustrup-Jensen MS, Sorensen TL, Petersen J, Andersen JP, Vilsen B, Nissen P. 2007. Crystal structure of the sodium-potassium pump. *Nature* **450**:1043–1049 DOI 10.1038/nature06419.

Nyblom M, Poulsen H, Gourdon P, Reinhard L, Andersson M, Lindahl E, Fedosova N, Nissen P. 2013. Crystal structure of Na^+, K(+)-ATPase in the Na(+)-bound state. *Science* **342**:123–127 DOI 10.1126/science.1243352.

Obara K, Miyashita N, Xu C, Toyoshima I, Sugita Y, Inesi G, Toyoshima C. 2005. Structural role of countertransport revealed in Ca2+ pump crystal structure in the absence of Ca2+. *Proceedings of the National Academy of Sciences of the United States of America* **102**:14489–14496 DOI 10.1073/pnas.0506222102.

Ogawa H, Cornelius F, Hirata A, Toyoshima C. 2015. Sequential substitution of K(+) bound to Na(+), K(+)-ATPase visualized by X-ray crystallography. *Nature Communications* **6**:8004 DOI 10.1038/ncomms9004.

Ogawa H, Shinoda T, Cornelius F, Toyoshima C. 2009. Crystal structure of the sodium-potassium pump (Na^+, K^+-ATPase) with bound potassium and ouabain. *Proceedings of the National Academy of Sciences of the United States of America* **106**:13742–13747 DOI 10.1073/pnas.0907054106.

Olesen C, Picard M, Winther A-ML, Gyrup C, Morth JP, Oxvig C, Nissen JV, Møller P. 2007. The structural basis of calcium transport by the calcium pump. *Nature* **450**:1036–1042 DOI 10.1038/nature06418.

Olesen C, Sørensen TL-M, Nielsen RC, Møller JV, Nissen P. 2004. Dephosphorylation of the calcium pump coupled to counterion occlusion. *Science* **306**:2251–2255 DOI 10.1126/science.1106289.

Ovchinnikov YA, Modyanov NN, Broude NE, Petrukhin KE, Grishin AV, Arzamazova NM, Aldanova NA, Monastyrskaya GS, Sverdlov ED. 1986. Pig kidney Na$^+$, K$^+$-ATPase: primary structure and spatial organization. *FEBS Letters* **201**:237–245 DOI 10.1016/0014-5793(86)80616-0.

Paulsen ES, Villadsen J, Tenori E, Liu H, Bonde DF, Lie MA, Bublitz M, Olesen C, Autzen HE, Dach I, Sehgal P, Nissen P, Møller JV, Schiøtt B, Christensen SB. 2013. Water-mediated interactions influence the binding of thapsigargin to sarco/endoplasmic reticulum calcium adenosinetriphosphatase. *Journal of Medicinal Chemistry* **56**:3609–3619 DOI 10.1021/jm4001083.

Pedersen PA, Jorgensen JR, Jorgensen PL. 2000. Importance of conserved alpha-subunit segment 709GDGVND for Mg2+ binding, phosphorylation, and energy transduction in Na, K-ATPase. *Journal of Biological Chemistry* **275**:37588–37595 DOI 10.1074/jbc.M005610200.

Pranata J, Wierschke SG, Jorgensen WL. 1991. OPLS potential functions for nucleotide bases. Relative association constants of hydrogen-bonded base pairs in chloroform. *Journal of the American Chemical Society* **113**:2810–2819 DOI 10.1021/ja00008a002.

Sacchetto R, Bertipaglia I, Giannetti S, Cendron L, Mascarello F, Damiani E, Carafoli E, Zanotti G. 2012. Crystal structure of sarcoplasmic reticulum Ca2+-ATPase (SERCA) from bovine muscle. *Journal of Structural Biology* **178**:38–44 DOI 10.1016/j.jsb.2012.02.008.

Šali A. 1995. Comparative protein modeling by satisfaction of spatial restraints. *Molecular Medicine Today* **1**:270–277 DOI 10.1016/S1357-4310(95)91170-7.

Šali A, Blundell TL. 1993. Comparative protein modelling by satisfaction of spatial restraints. *Journal of Molecular Biology* **234**:779–815 DOI 10.1006/jmbi.1993.1626.

Schoner W, Beusch R, Kramer R. 1968. On the mechanism of Na plus and K plus-stimulated hydrolysis of adenosine triphosphate. 2. Comparison of nucleotide specificities of Na plus and K plus-activated ATPase and Na plus-dependent phosphorylation of cell membranes. *European Journal of Biochemistry* **7**:102–110 DOI 10.1111/j.1432-1033.1968.tb19580.x.

Shinoda T, Ogawa H, Cornelius F, Toyoshima C. 2009. Crystal structure of the sodium-potassium pump at 2.4 A resolution. *Nature* **459**:446–450 DOI 10.1038/nature07939.

Shull MM, Pugh DG, Lingrel JB. 1989. Characterization of the human Na, K-ATPase alpha 2 gene and identification of intragenic restriction fragment length polymorphisms. *Journal of Biological Chemistry* **264**:17532–17543.

Shull GE, Schwartz A, Lingrel JB. 1985. Amino-acid sequence of the catalytic subunit of the (Na$^+$ + K$^+$) ATPase deduced from a complementary DNA. *Nature* **316**:691–695 DOI 10.1038/316691a0.

Sohoel H, Jensen AM, Moller JV, Nissen P, Denmeade SR, Isaacs JT, Olsen CE, Christensen SB. 2006. Natural products as starting materials for development of second-generation SERCA inhibitors targeted towards prostate cancer cells. *Bioorganic and Medicinal Chemistry* **14**:2810–2815 DOI 10.1016/j.bmc.2005.12.001.

Sonntag Y, Musgaard M, Olesen C, Schiøtt B, Møller JV, Nissen P, Thøgersen L. 2011. Mutual adaptation of a membrane protein and its lipid bilayer during conformational changes. *Nature Communications* **2**:304 DOI 10.1038/ncomms1307.

Sorensen TL, Moller JV, Nissen P. 2004. Phosphoryl transfer and calcium ion occlusion in the calcium pump. *Science* **304**:1672–1675 DOI 10.1126/science.1099366.

Takahashi M, Kondou Y, Toyoshima C. 2007. Interdomain communication in calcium pump as revealed in the crystal structures with transmembrane inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **104**:5800–5805 DOI 10.1073/pnas.0700979104.

Tejral G, Kolácná L, Kotyk A, Amler E. 2007. Comparative modeling of the H_4–H_5-loop of the α_2-isoform of Na^+/K^+-ATPase α-subunit in the E_1 conformation. *Physiological Research/Academia Scientiarum Bohemoslovaca* **56(Suppl 1)**:S143–S151.

Tejral G, Kolácná L, Schoner W, Amler E. 2009. The π-helix formation between Asp^{369} and Thr^{375} as a key factor in E_1–E_2 conformational change of Na^+/K^+-ATPase. *Physiological Research/Academia Scientiarum Bohemoslovaca* **58**:583–589.

Toyoshima C. 2008. Structural aspects of ion pumping by Ca2+-ATPase of sarcoplasmic reticulum. *Archives of Biochemistry and Biophysics* **476**:3–11 DOI 10.1016/j.abb.2008.04.017.

Toyoshima C, Iwasawa S, Ogawa H, Hirata A, Tsueda J, Inesi G. 2013. Crystal structures of the calcium pump and sarcolipin in the Mg2+-bound E1 state. *Nature* **495**:260–264 DOI 10.1038/nature11899.

Toyoshima C, Mizutani T. 2004. Crystal structure of the calcium pump with a bound ATP analogue. *Nature* **430**:529–535 DOI 10.1038/nature02680.

Toyoshima C, Nakasako M, Nomura H, Ogawa H. 2000. Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. *Nature* **405**:647–655 DOI 10.1038/35015017.

Toyoshima C, Nomura H. 2002. Structural changes in the calcium pump accompanying the dissociation of calcium. *Nature* **418**:605–611 DOI 10.1038/nature00944.

Tran CM, Farley RA. 1999. Catalytic activity of an isolated domain of Na, K-ATPase expressed in *Escherichia coli*. *Biophysical Journal* **77**:258–266 DOI 10.1016/S0006-3495(99)76887-6.

Trott O, Olson AJ. 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry* **31**:455–461 DOI 10.1002/jcc.21334.

Van der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJC. 2005. GROMACS: fast, flexible, and free. *Journal of Computational Chemistry* **26**:1701–1718 DOI 10.1002/jcc.20291.

Van der Spoel D, Lindahl E, Hess B, Van Buuren AR, Apol E, Meulenhoff PJ, Tieleman DP, Sijbers ALTM, Feenstra KA, Van Drunen R, Berendsen HJC. 2010. Gromacs user manual version 4.5.6. *Available at ftp://ftp.gromacs.org/pub/manual/manual-4.5.6.pdf*.

Winther AM, Bublitz M, Karlsen JL, Moller JV, Hansen JB, Nissen P, Buch-Pedersen MJ. 2013. The sarcolipin-bound calcium pump stabilizes calcium sites exposed to the cytoplasm. *Nature* **495**:265–269 DOI 10.1038/nature11900.

Winther A-ML, Liu H, Sonntag Y, Olesen C, Maire Ml, Soehoel H, Olsen C-E, Christensen SB, Nissen P, Møller JV. 2010. Critical roles of hydrophobicity and orientation of side chains for inactivation of sarcoplasmic reticulum Ca2+-ATPase with thapsigargin and thapsigargin analogs. *Journal of Biological Chemistry* **285**:28883–28892 DOI 10.1074/jbc.M110.136242.

Yatime L, Laursen M, Morth JP, Esmann M, Nissen P, Fedosova NU. 2011. Structural insights into the high affinity binding of cardiotonic steroids to the Na^+, K^+-ATPase. *Journal of Structural Biology* **174**:296–306 DOI 10.1016/j.jsb.2010.12.004.